Homozygous Variegate Porphyria: Identification of Mutations on Both Alleles of the Protoporphyrinogen Oxidase Gene in a Severely Affected Proband

Jorge Frank, John McGrath,* HaMut Lam, Robert M. Graham,† John L. M. Hawk,‡ and Angela M. Christiano

Department of Dermatology, Columbia University, New York, New York, U.S.A.; *St. John's Hospital, London, U.K.; †Department of Dermatology, James Paget NHS Trust, Great Yarmouth, U.K.; ‡St. John's Institute of Dermatology, St. Thomas Hospital, London, U.K.

Homozygous variegate porphyria is a severe skin and neurologic disease manifesting in early infancy, and characterized by markedly reduced levels of the penultimate enzyme in the heme biosynthetic pathway, protoporphyrinogen oxidase. We investigated the molecular basis of variegate porphyria, usually an autosomal dominantly inherited trait, in a severely affected female proband and her parents. The mutation detection strategy included heteroduplex analysis, automated sequencing, and allele specific oligonucleotide hybridization. We identified two underlying missense mutations in the protoporphyrinogen oxidase gene, consisting of a G-to-A transition in exon 6 (G169E), and a G-to-A transition in exon 10 (G358R). Our study establishes the molecular basis of "homozygous" variegate porphyria for the first time, in demonstrating that this patient is a compound heterozygote for two different missense mutations in the protoporphyrinogen oxidase gene. ***Key words: genodermatosis/photosensitivity/porphyria/porphyrin-heme biosynthetic pathway. J Invest Dermatol 110:452–455, 1998***

The porphyrias are disorders of heme metabolism resulting from the inherited or acquired dysregulation of one of the eight enzymes that control the porphyrin-heme biosynthetic pathway. Variegate porphyria (VP), one of the acute hepatic porphyrias, is characterized by a partial defect in the activity of protoporphyrinogen oxidase (PPO), the penultimate enzyme in the heme biosynthetic pathway (Frank and Christiano, 1997).

VP is usually inherited as an autosomal dominant trait, displaying incomplete penetrance, as not all persons carrying a mutation in the PPO gene develop the clinical phenotype (Frank and Christiano, 1997). In heterozygotes, PPO activity is decreased by ≈50%. Homozygous VP is a very rare recessive disease, presenting with residual PPO levels between 5 and 20%, and to date only a few cases have been reported (Kordac *et al*, 1984; Murphy *et al*, 1986; Mustajoki *et al*, 1987; Norris *et al*, 1990; Coakley *et al*, 1990; Gandolfo *et al*, 1991; Hift *et al*, 1993).

Clinically, VP is characterized by cutaneous manifestations, including increased photosensitivity, blistering, skin fragility with chronic scarring of sun-exposed areas, and postinflammatory hyperpigmentation (Dean, 1971). Acute exacerbations of VP include abdominal pain, the passage of dark urine, and neuropsychiatric symptoms that characterize the acute hepatic porphyrias, such as bulbar paralysis, quadriplegia, motor neuropathy, and weakness of the limbs (Frank and Christiano, 1997). In heterozygotes, the disease does not usually present before puberty (Coakley *et al*, 1990), whereas homozygous patients develop severe symptoms in early infancy (Hift *et al*, 1993).

The human PPO cDNA and gene were recently cloned and mapped to chromosome 1q22–23 (Nishimura *et al*, 1995; Roberts *et al*, 1995; Taketani *et al*, 1995a), and we and others have recently identified pathogenetic mutations in several families with VP (Deybach *et al*, 1996; Meissner *et al*, 1996; Warnich *et al*, 1996; Lam *et al*, 1997; Frank and Christiano, 1997). In this study, we investigated a proband who was diagnosed with "homozygous" VP in 1989 (Norris *et al*, 1990), and her clinically unaffected parents. We identified a G-to-A transition in exon 6 and a G-to-A transition in exon 10 of the PPO gene, resulting in the substitution of glycine by glutamic acid (G169E) on the paternal PPO allele, and glycine by arginine (G358R) on the maternal allele, respectively. Our study establishes, for the first time, that this patient is a compound heterozygote for two different missense mutations in the PPO gene and thereby offers a molecular explanation for the autosomal recessive "homozygous" form of VP.

MATERIALS AND METHODS

Probands and controls The patient, her parents, and 50 unrelated unaffected controls were investigated in this study. Diagnosis of VP in the patient was established by one of us (J.L.M. Hawk) in a previously published investigation (Norris *et al*, 1990). The now 20 y old female proband was the daughter of nonconsanguineous parents with no family history of porphyria.

Clinical material Blood samples from the proband (**Fig 1*A***) and her parents were collected in tubes containing ethylenediamine tetraacetic acid. All individuals provided informed consent for inclusion in the investigation, in accordance with guidelines set forth by the local institutional review board. Genomic DNA was isolated according to standard techniques (Sambrook *et al*, 1989).

Polymerase chain reaction (PCR) amplification and mutation detection A mutation detection strategy was developed for PCR amplification of all PPO exons using PCR primers that were published recently (Warnich *et al*, 1996). For amplification of exon 6 of the PPO gene, the following primers were used: PPO exon 6L, 5′GGGCTGTGGAAATCAGTCAG3′; and PPO exon 6R, 5′TTCACCCTCTGAATCGATCC3′. For amplification of exon 10 of the PPO gene, the following primers were used: PPO exon 10L,

Manuscript received May 19, 1997; revised November 12, 1997; accepted for publication November 24, 1997.

Reprint requests to: Dr. Angela M. Christiano, Department of Dermatology, Columbia University, College of Physicians and Surgeons, 630 West 168th Street, VC-1526, New York, New York 10032.

Abbreviations: PPO, protoporphyrinogen oxidase; VP, variegate porphyria.

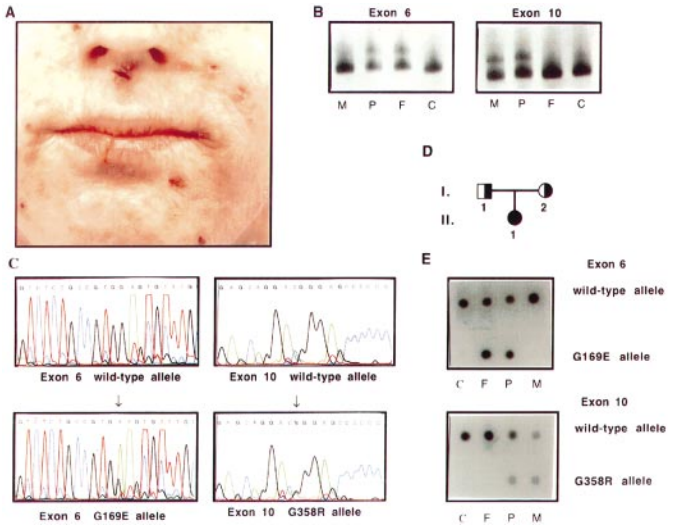

Figure 1. Mutation analysis in the VP patient in this study and her family members. (*A*) Clinical findings in the patient on sun-exposed area; erosions, crusts, superficial scars, perioral radial linear scarring. (*B*) Heteroduplex analysis of the family and a control (C); heteroduplex formation in the proband (P) and her father (F) in exon 6, and the proband (P) and her mother (M) in exon 10 of the PPO gene. (*C*) Automated sequence of the forward strands of the PCR fragments of exon 6 and exon 10, respectively; G-to-A transition in exon 6 of the proband (*lower panel*, indicated by an arrow) compared with the control (*upper panel*), and G-to-A transition in exon 10 of the proband (*lower panel*, indicated by an arrow) compared with the control (*upper panel*). (*D*) Pedigree of the nuclear family; the proband (II/1) is indicated by a filled circle, her parents (I/1 and I/2) are indicated by half-filled symbols. (*E*) ASO hybridization results: in exon 6, the proband (P) and her father (F) show the mutant allele G169E (*lower panel*) and the wild-type allele, whereas mother (M) and control (C) only show wild-type allele (*upper panel*); in exon 10, proband (P) and her mother (M) show the mutant allele G358R (*lower panel*) and the wild-type allele, whereas father (F) and control (C) only show the wild-type allele (*upper panel*).

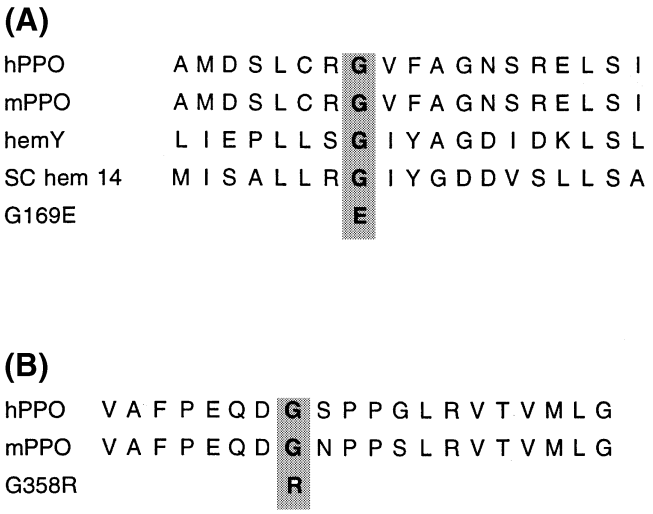

Figure 2. (*A*) Alignment of deduced amino acid sequences from codons 161 to 180 in exon 6 of human PPO (hPPO), mouse PPO (mPPO), the Hem Y gene product from *Bacillus subtillis* (hemY), and the yeast *Saccharomyces cerevisiae* (SC hem 14). The identical residues in codon 169 and the substitution derived from the described missense mutation are shaded and bold. (*B*) Alignment of deduced amino acid sequences from codons 351 to 370 in exon 10 of human PPO (hPPO) and mouse PPO (mPPO). The identical residues in codon 358 and the substitution derived from the described missense mutation are shaded and bold.

5′AGAGCCCTTTCCTTCTGACGCATG3′; and PPO exon 10R, 5′TGGCCTTGCCTACAATGGAGCAC3′.

PCR was carried out on genomic DNA from all family members according to the following program: 95°C for 10 min; followed by 40 cycles of 95°C for 45 s, 57°C for 45 s, and 72°C for 1 min; followed by 72°C for 15 min, in an OmniGene thermal cycler (Marsh Scientific, Rochester, NY). For mutation detection, PCR products were subjected to conformation sensitive gel electrophoresis (CSGE) analysis as described previously (Ganguly *et al*, 1993). PCR products displaying a heteroduplex on CSGE analysis were sequenced automatically using an ABI Prism 310 Genetic Analyzer. To verify the mutations, allele specific oligonucleotide hybridization was performed according to standard techniques (Sambrook *et al*, 1989). In this case, the following oligonucleotides were used: Oligo exon 6 mutant, 5′TCTGCCGT*GAA*GTGTTTGCA3′; and Oligo exon 6 wild-type, 5′TCTGCCGT*GGA*GTGTTTGC3′. Oligo exon 10 mutant, 5′AGCAGGAC*AGG*AGCCCC3′; and Oligo exon 10 wild-type, 5′GCAGGAC*GGG*AGCCCC3′.

RESULTS

Heteroduplex analysis of exon 6 of the PPO gene in this family revealed a heteroduplex in the proband and her father (**Fig 1*B***). Automated sequencing of the PCR product of exon 6 revealed a similar sequence deviation in the proband and her father (**Fig 1*C***). The mutation consisted of a G-to-A transition at nucleotide 505 of the PPO cDNA (numbered according to GenBank #D38537). This base substitution leads to a missense mutation consisting of an amino acid change from glycine to glutamic acid at position 169 (G169E) in the deduced amino acid sequence on the paternal allele.

Heteroduplex analysis of exon 10 of the PPO gene in this family revealed a heteroduplex in the proband and her mother (**Fig 1*B***). Automated sequence analysis of the PCR product of exon 10 showed the same sequence variant in the proband and her mother (**Fig 1*C***). The mutation consisted of a G-to-A transition at nucleotide 1071 of the PPO cDNA (numbered according to GenBank #D38537). This base change results in a missense mutation causing an amino acid substitution from glycine to arginine at position 358 (G358R) in the deduced amino acid sequence on the maternal allele.

Heteroduplex analysis of exon 6 and exon 10 of the PPO gene was also carried out in 50 unrelated control individuals and showed no heteroduplex formation indicative of the mutation, suggesting that these sequence variations are not common polymorphisms in the PPO gene (data not shown).

To verify the sequence variations in exon 6 and exon 10, allele specific oligonucleotide hybridization was performed (**Fig 1*E***). Furthermore, all remaining PPO exons of the patient and her parents were checked for additional sequence variants but revealed no other mutations.

The proband revealed raised erythrocyte protoporphyrin levels with the protoporphyrin being predominantly zinc-chelated (Norris *et al*, 1990) and PPO activity measured in Epstein–Barr virus transformed lymphoblasts was decreased to less than 20%, whereas both parents had approximately half-normal levels of PPO (Norris *et al*, 1990) (**Table I**).

DISCUSSION

Only 10 patients with the diagnosis of "homozygous" VP have been reported (Kordac *et al*, 1984; Murphy *et al*, 1986; Mustajoki *et al*, 1987; Norris *et al*, 1990; Coakley *et al*, 1990; Gandolfo *et al*, 1991; Hift *et al*, 1993), indicating that this variant of VP is very rare. These 10 cases were reported at a time when the genetic lesions in the PPO gene were not yet identified, so that making a DNA based diagnosis was not possible. Therefore, at that time, diagnosis of "homozygous" VP was made by the severe clinical findings in the patients and biochemical analyses; however, Hift *et al* postulated in 1993 that some of the cases of "homozygous" VP would be compound heterozygotes rather than true homozygotes (Hift *et al*, 1993). In support of this notion, our results demonstrate that the "homozygous" VP patient reported by Norris *et al* (1990) is indeed a compound heterozygote.

Twelve mutations have been identified to date in the PPO gene (**Table II**) (Deybach *et al*, 1996; Meissner *et al*, 1996; Warnich *et al*, 1996; Lam *et al*, 1997; Frank and Christiano, 1997). The paternal mutation detected in this study is a G-to-A transition in exon 6 of the PPO gene, designated G169E. Three lines of evidence support the notion that this base substitution is responsible for VP in this severely affected proband. First, it causes an amino acid change from a neutral glycine residue to a negatively charged glutamic acid residue at position

Table I. Results of measurement of porphyrins and their precursors in the patient's urine, stool, erythrocytes lymphoblasts according to Norris *et al* (1990)

	Proband	Father	Mother	Reference ranges
Urine				
Uroporphyrin (μg per liter)	145	–	–	0–18
Coproporphyrin (μg per liter)	184	–	–	0–149
Stool				
Coproporphyrin (μg per g dry weight)	131	8	13	0–30
Protoporphyrin (μg per g dry weight)	171	12	17	0–125
Ether insoluble (X) porphyrin (μg per dry weight)	332	5	5	0–20
Erythrocytes				
Total porphyrin (μg per dl)	645	74	121	0–95
Lymphoblasts				
PPO enzyme activity (nmol protoporphyrin per hour per mg protein)	0.06	0.28	0.29	0.38–0.47

Table II. Mutations in the PPO gene reported to date

Designation	Location	Consequence	Reference
Dominant VP			
1083delT	exon 10	Frameshift mutation	Frank and Christiano, 1997
165insAG	exon 3	Frameshift mutation	Lam *et al*, 1997
1144del GT	exon 11	Frameshift mutation	Frank and Christiano, 1997
1022insG	exon 7	Frameshift mutation	Deybach *et al*, 1996
S450P	exon 13	Missense mutation	Frank and Christiano, 1997
G232R	exon 7	Missense mutation	Deybach *et al*, 1996
H20P	exon 2	Missense mutation	Warnich *et al*, 1996
R59W	exon 3	Missense mutation	Meissner *et al*, 1996 Warnich *et al*, 1996
R168C	exon 6	Missense mutation	Meissner *et al*, 1996 Warnich *et al*, 1996
E133X	exon 5	Nonsense mutation	Frank *et al*, 1998
Recessive VP			
G169E/G358R	exon 6/exon 10	Compound heterozygote missense mutation	This study

169 (G169E) on the paternal allele of the PPO gene that may destabilize specific interactions in PPO and impair proper functioning of the protein. Second, a comparison of deduced amino acid sequences (**Fig 2*A***) revealed this glycine residue to be strictly conserved through evolution in human (Nishimura *et al*, 1995), mouse (Taketani *et al*, 1995b), *Bacillus subtilis* (Taketani *et al*, 1995b), and *Saccharomyces cerevisiae* (Camadro and Labbe, 1996), although their entire amino acid sequence identity is less than 20%. Third, no other deviation in the father's cDNA sequence of the PPO gene was detected.

The maternal mutation consisted of a G-to-A transition in exon 10 of the PPO gene, designated G358R. Because glycine is a neutral amino acid whereas arginine is positively charged, this mutation is also likely to cause disturbances and dysfunction of the protein. A comparison of deduced amino acid sequences (**Fig 2*B***) showed that this glycine residue is conserved in human and mouse PPO, over 90 million evolutionary years. No other abnormality was found in the coding sequence of the mother's PPO gene.

Usually, VP is inherited in an autosomal dominant fashion (Frank and Christiano, 1997). In this study, however, we demonstrated that disease transmission in this family is clearly autosomal recessive. As each parent carried one mutation and had a reduced enzymatic activity (**Table I**), each of the mutations must reduce the activity of PPO; however, family history did not reveal any clinical symptoms of VP. It is not yet understood why some heterozygous carriers of mutations in the PPO gene develop the clinical phenotype and others do not.

In this case, the affected proband was a compound heterozygote for the mutations and revealed markedly reduced PPO activity (<20%) and severe clinical symptoms (Norris *et al*, 1990). Thus, we postulate that inheritance of two missense mutations leads to a profound biochemical defect that predisposes to a severe phenotype with clinical findings that are not present in heterozygous VP patients. The clinically severe recessive form of VP described here has similarities to the "homozygous" forms of erythropoietic protoporphyria, hereditary coproporphyria, and porphyria cutanea tarda reported previously (Grandchamp *et al*, 1977; Elder *et al*, 1981; Goerz *et al*, 1994; Sarkany and Cox, 1995). It appears that such a pattern of disease inheritance is associated with a more severe clinical phenotype. Remarkably, only in the reported cases with "homozygous" VP does the clinical phenotype include mental retardation, hand deformities, developmental delay, and nystagmus (Hift *et al*, 1993), presumably as a consequence of the profound enzyme deficiency. We propose that probands with this recessively inherited variant of VP are at high risk for the development of more severe clinical findings.

Interestingly, only "homozygous" VP patients show elevated protoporphyrin levels in the erythrocytes, with the protoporphyrin being predominantly zinc-chelated (Hift *et al*, 1993), whereas heterozygous VP patients do not. This accumulation of protoporphyrin was also reported in cases of homozygous porphyria cutanea tarda (Elder *et al*, 1981) and in homozygous hereditary coproporphyria (Nordmann *et al*, 1982), and appears to be a general consequence of severe enzymatic defects in the porphyrin-heme biosynthetic pathway.

This study establishes the molecular basis of "homozygous" VP for the first time. Further investigations are underway to elucidate the molecular basis of VP, establish genotype-to-phenotype correlations by the expression of mutant alleles, and understand additional and unusual mechanisms of inheritance of the disease. These studies will provide the basis for the design of enzyme-replacement strategies as a therapy for VP in the future.

We are especially grateful to the patient and her family for their interest and cooperation in this study. We gratefully acknowledge the contribution of Professor G.H. Elder (Department of Medical Biochemistry, University of Wales College of Medicine, Cardiff, U.K.) to the initial biochemical assessment of our patient's porphyria. This study was supported by Grants FR 1315/1–1 (J.F.) from the Deutsche Forschungsgemeinschaft (DFG) and the American Porphyria Foundation (A.M.C.).

REFERENCES

Camadro JM, Labbe P: Cloning and characterization of the yeast HEM14 coding for protoporphyrinogen oxidase, the molecular target of diphenyl ether-type herbicides. *J Biol Chem* 271:9120–9128, 1996

Coakley J, Hawkins R, Crinis N, *et al*: An unusual case of variegate porphyria with possible homozygous inheritance. *Aust NZ J Med* 20:587–589, 1990

Dean G: *The Porphyrias*, 2nd edn. Pitman Medical, London, 1971

Deybach JC, Puy H, Robreau AM, Lamoril J, Da Silva V, Grandchamp B, Nordmann Y: Mutations in the protoporphyrinogen oxidase gene in patients with variegate porphyria. *Hum Mol Genet* 5:407–410, 1996

Elder GH, Smith SG, Herrero C, *et al*: Hepatoerythropoietic protoporphyria: a new uroporphyrinogen decarboxylase defect or homozygous porphyria cutanea tarda? *Lancet* i:916–919, 1981

Frank J, Christiano AM: Genetic research strategies: a review of the acute porphyrias. *Retinoids* 13:88–92, 1997

Frank J, Jugert FK, Kalka K, Goerz G, Merk HF, Christiano AM: Variegate porphyria: identification of a nonsense mutation in the protoporphyrinogen oxidase gene. *J Invest Dermatol* 110:449–451, 1998

Gandolfo LD, Macri A, Biolcati G, *et al*: Homozygous variegate porphyria: revision of a diagnostic error. *Br J Derm* 124:211, 1991

Ganguly A, Rock MJ, Prockop DJ: Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: Evidence for solvent-induced bends in DNA heteroduplexes. *Proc Natl Acad Sci USA* 90:10325–10329, 1993

Goerz G, Bolsen K, Bunselmeyer S, Schurer NY: Recessive inheritance of erythropoietic protoporhyria with liver failure. *Lancet* 344:337, 1994

Grandchamp B, Phung N, Nordmann Y: Homozygous case of hereditary coproporhyria. *Lancet* ii:1348–1349, 1977

Hift RJ, Meissner PN, Todd G, *et al*: Homozygous variegate porphyria: an evolving clinical syndrome. *Postgrad Med J* 69:781–786, 1993

Kordac V, Deybach JCH, Martasek P, *et al*: Homozygous variegate porphyria. *Lancet* 11:851, 1984

Lam H, Dragan L, Tsou HC, *et al*: Molecular basis of variegate porphyria: A *de novo* insertion mutation in the protoporphyrinogen oxidase gene. *Hum Genet* 99:126–129, 1997

Meissner PN, Dailey TA, Hift RJ, *et al*: A R59W mutation in human protoporphyrinogen oxidase results in decreased enzyme activity and is prevalent in South Africans with variegate porphyria. *Nat Genet* 13:95–97, 1996

Murphy GM, Magnus IA, Barrett DR, Elder GH, Hawk JLM, Smith SG: Homozygous variegate porphyria: two similar cases in unrelated families. *J R Soc Med* 79:361–363, 1986

Mustajoki P, Tenhunen R, Niemi KM, Nordmann Y, Kääriäinen H, Norio R: Homozygous variegate porphyria: A severe skin disease of infancy. *Clin Genet* 32:300–305, 1987

Nishimura K, Taketani S, Inokuchi H: Cloning of a human cDNA for protoporphyrinogen oxidase by complementation *in vivo* of a *hem* G mutant of *Escherichia coli*. *J Biol Chem* 270:8076–8080, 1995

Nordmann Y, Grandchamp B, de Verneuil H: Hardero-porphyria: a variant hereditary coproporphyria. *J Clin Invest* 72:1139–1143, 1982

Norris PG, Elder GH, Hawk JLM: Homozygous variegate porphyria: a case report. *Br J Derm* 122:253–257, 1990

Roberts AG, Whatley SD, Daniels J, *et al*: Partial characterization and assignment of the gene for protoporphyrinogen oxidase and variegate porphyria to human chromosome 1q23. *Hum Molec Genet* 4:2387–2390, 1995

Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York, 1989

Sarkany RPE, Cox TM: Autosomal recessive erythropoietic protoporphyria: a syndrome of severe photosensitivity and liver failure. *Q J Med* 88:541–549, 1995

Taketani S, Inazawa J, Abe T, *et al*: The human protoporphyrinogen oxidase gene (PPOX): Organization and location to chromosome 1. *Genomics* 29:698–703, 1995a

Taketani S, Yoshinaga T, Furukawa T, Kohno H, Tokunaga R, Nishimura K, Inokuchi H: Induction of terminal enzymes for heme biosynthesis during differentiation of mouse erythroleukemia cells. *Eur J Biochem* 230:760–765, 1995b

Warnich L, Kotze MJ, Groenewald IM, *et al*: Identification of three mutations and associated haplotypes in the protoporphyrinogen oxidase gene in South African families with variegate porphyria. *Hum Mol Genet* 5:981–984, 1996